Evaluation of four cell lines for assay of infectious adenoviruses in water samples

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ABSTRACT

Human viral contamination in drinking and recreational waters poses health risks. The application of PCR-based molecular technology has advanced our knowledge of the occurrence and prevalence of human viruses in water; however, it has provided no information on viral viability and infectivity. Four human cell lines were compared for their sensitivity to different serotypes of human adenoviruses using the $TCID_{50}$ test. The sensitivity of each cell line varied with different serotypes of adenovirus. Human embryonic kidney cell line 293A and human lung carcinoma cell line A549 were the most sensitive, especially to enteric adenovirus 40 and 41. Plaque assay of primary sewage samples showed 293A can detect viral plaques in 7 of 13 primary sewage samples tested. Adenoviruses were also isolated using 293A from environmental water concentrates. Cloning and sequencing of environmental adenoviral isolates indentified them to be aligned with adenoviruses serotype 40 and serotype 5. The result of this study suggests that plaque assay with 293A cell line is suitable for detection of adenovirus in the aquatic environment. Combining this cell culture with molecular methods for viral assay in the aquatic environment will provide critical information for risk assessment.

Key words | adenovirus, aquatic, infectivity, plaque assay, tissue culture

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INTRODUCTION

Human viral contamination of drinking and recreational waters poses an important human health risk. To prevent human exposures, it is necessary to monitor source waters used for drinking water supply and recreational waters for viral contamination. In recent years, molecular approaches (e.g. PCR) for viral detection have overtaken the traditional cell culture assay because of their improved speed and sensitivity. However, these molecular methods often yield little or no information on viral viability and infectivity; thus, they are often insufficient for regulatory and policy decisions. In contrast with the attention paid to molecular technologies, little energy has been applied in testing and improving the traditional cell culture for detecting human viruses in the environment. Important enteric viruses (i.e. noroviruses), which cause recurring outbreaks, have not yet been cultivable.

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In the past few years, our lab has focused on using adenoviruses (Ad) as indicators for human viral pollution in the aquatic system. Application of PCR methods has initially revealed the presence and prevalence of Ad in the Los Angeles and San Gabriel Rivers, California. However, no infectious virus has been isolated on cell culture from the rivers (Choi & Jiang 2005). Adenoviruses can be cultured and isolated using various cell lines including BGMK, Caco-2, HeLa, HEp-2, KB (human oral epidermoid), A549 (human lung carcinoma), PLC/PRF/5 and HEK 293 (human embryonic kidney). However, the efficiency of viral replication on cell cultures varies with serotypes. The enteric Ad serotype 40 and 41 are very slow growing and produce little or no cytopathic effect (CPE) on most cell lines. Mautner *et al.* (1989) showed that only cell lines expressing Ad E1A and that supply Ad E1B functions are

permissive for Ad40. HEK Graham 293 is a permanent cell line transformed by sheared human Ad type 5 (Ad5) DNA. The cells express the viral proteins from transforming gene of Ad5 including E1A and E1B (Graham *et al.* 1977). For this reason, Graham 293 cells support the propagation of Ad40 and Ad41.

A number of studies have been done to evaluate the efficiency of various cell lines in support of clinical Ads replication (see review in Jiang 2006). The susceptibility of the cell lines may vary slightly with sample sources and labs, but the literature generally shows a consensus that 293, A549, PLC/PRF/5 and Caco-2 performed better for Ads than BGMK, the cell line recommended by USEPA for water monitoring. A review of current literature on Ads detection in aquatic environments indicates that BGMK was most often the choice for the cell culture (Chapron *et al.* 2000; Cho *et al.* 2000; Thompson *et al.* 2003; Lee & Jeong 2004; Lee *et al.* 2005). Thus, the occurrence of infectious Ads in the aquatic environment is possibly underestimated using the current USEPA Information Collection Rule, especially for the fastidious Ad40 and 41. So far, to our knowledge, there has not been an investigation of cell lines that best support infectivity of Ads from aquatic samples.

Here we compared four different human cell lines for the sensitivity to human Ad serotype 2, 15, 40 and 41. The best cell lines were then chosen to test environmental samples collected from sewage treatment facilities and various water samples from different areas of southern California. Environmental adenoviral isolates were further confirmed by DNA sequencing. This study contributes to our ability to detect and identify Ad pollution in the aquatic environment.

MATERIALS AND METHODS

Viruses, cell lines and culture condition

Adenovirus serotype 2 (Ad2), 5 (Ad5) and 15 (Ad15) were obtained from University of Southern California (courtesy of professor Michael Lai). Ad5 is a recombinant virus with E1 gene replaced by a green fluorescent protein (GFP) gene. This virus was used as a positive control for the plaque assay. Adenovirus serotype 40 (Ad40) was isolated from an environmental sample from Newport Bay, California, by cell culture (detailed below) in our laboratory and confirmed by sequencing of a 482-bp region in the hexon gene. Ad41 was provided by Dr Shawn Thompson at Los Angeles Sanitation District.

The human embryonic kidney cell line (HEK-293A) and human hepatoma cell lines (HuH7 and HepG2) were also obtained from the University of Southern California (courtesy of Professor Michael Lai) and were used in this study between passage 42 and 50. HEK-293A is a mutant of Graham 293 with better adhesive ability to the culture surface (Michael Lai, personal communication). The cells were grown at 37°C in Dulbecco's modified Eagle's medium with glucose and L-glutamine and supplemented with 5% fetal bovine serum (FBS), 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin. Human lung carcinoma cell line A549 was obtained from Los Angeles Sanitation District (courtesy of Dr Shawn Thompson) at passage 108 and was used in this study between passage 115 and 123. A549 cells were grown in Ham's F12 medium also containing 5% FBS, 2 mM L-glutamine, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin. Cells were subcultured at 4- to 5-day intervals with a trypsin-EDTA solution (Cellgro).

Infective assay ($TCID_{50}$)

Purified Ad2, Ad15, Ad40 and Ad41 viruses were released from infected cells by freeze and thaw cycles. The viral particles were concentrated by ultracentrifugation and then washed twice with pH 7.4 PBS (phosphate buffered saline). Infectivity and sensitivity of these viruses on each cell line were determined in microtitre plates by serial dilution of viral stock and inoculation 100 μl of dilution into each of the 96 wells containing monolayer of 293A, A549, HuH7 or HepG2 cells, respectively. Plates were incubated at 37°C with 5% CO_2 and examined daily for CPE for 10 days. $TCID_{50}$ values were calculated by the method of Reed & Muench (1938).

For 100 μl of dilution, the titre is: $T = 10^{1+d(S\,-\,0.5)}$, where d = log 10 of dilution and S = the sum of the ratios of positive wells per row.

The lower limit of detection for this assay is 102 $TCID_{50}$ ml^{-1}. Based on empirical assay, the titre by $TCID_{50}$ is 0.7 log higher than the titre by standard plaque assay (Reed & Muench 1938).

Environmental samples

Sewage samples were collected from three Southern California sewage treatment plants. Both primary sewage effluent and samples containing mixed primary and secondary effluent were tested for Ads. Sewage samples (10 ml to 30 ml) were ultracentrifuged at 210,000 × *g* for 90 min. Pellets were resuspended in 500 μl PBS (pH 7.4) and extracted with an equal volume of chloroform twice to remove cytotoxicants. The aqueous layer was further clarified by filtering through 0.2 μm pore size, low protein binding filters (PES, Whatman) to remove interferences and bacteria before inoculation.

Environmental water samples collected during several previous studies were tested for the presence of infectious human Ads. These samples were concentrated by ultrafiltration for PCR detection of human viruses in the previous studies, and were archived in −80°C freezers. A total of 466 frozen samples were used for infectivity tests. These included samples collected from the Los Angeles and San Gabriel Rivers during a seasonal study between February 2002 and February 2003 (Choi & Jiang 2005); storm drain and street gutter water samples collected in a small urban watershed in southern California (Jiang *et al.* 2007*b*); water samples collected from upstream Santa Ana River and a tributary during three consecutive storms (Surbeck *et al.* 2006); water samples collected from 15 locations around Newport Bay watershed between May 2002 and April 2003 (Jiang *et al.* 2007*a*); and costal waters collected at the mouth of Santa Ana River in summer 2001. The detailed sampling procedure and viral concentration methods can be found in the above publications listed in the References. The same purification procedures as for sewage samples were used to remove cytotoxicants from the concentrates.

Plaque assay of environmental samples

To assay Ad in aquatic samples, purified sewage and concentrated aquatic samples were inoculated onto confluent HEK- 293A and A549 cells in 6-well plates. The plates were incubated in a CO_2 incubator at 37°C for 1 h and were gently rocked every 15 min for viral adsorption. Then, inoculated cells were carefully washed once with pre-warmed PBS and overlaid with warm Dulbecco's modified Eagle's medium, 5% FBS, and 1.25% agarose containing 50 μg ml^{-1} gentamicin and 0.25 μg ml^{-1} amphotericin B. Cells were incubated at 37°C with 5% CO_2. A second overlay was applied at 6 to 7 days post-infection. Inoculated cell cultures were examined microscopically daily up to 2 weeks post-infection. Plaques were counted as PFU/100 ml sample at 10 days. Highly diluted recombinant Ad5 was used on HEK-293A cells as a positive control, while PBS was used as a negative control. The recombinant adenovirus expresses green firefly protein in infected cells; hereby, the resultant plaque can be confirmed and differentiated under UV microscopy.

Cloning and sequencing of environmental adenovirus

Samples showing signs of viral infection were scraped from the culture well and resuspended in PBS buffer. Viruses were released from cells by freeze and thaw cycles in liquid nitrogen. The cell debris was removed by low speed centrifugation. The supernatant was concentrated using ultracentrifugation at 210,000 × *g* for 90 min, and the pellet was resuspended and used for viral genome extraction using Qiagen Viral RNA mini Kit (Qiagen Inc.; recovers both RNA and DNA effectively). Viral genome was amplified using degenerate primers AD1 and AD2 specific to hexon gene region of diverse serotypes as reported in Xu *et al.* (2000). The resultant 482-bp amplicon was cloned into the pCR2.1-TOPO vector (Invitrogen Inc.). Sequencing was performed using primers flanking both ends of the plasmid insertion and was carried out with the ABI PRISM BigDye version 3.0 sequencing chemistry (Applied Biosystems, Foster City, California) by capillary electrophoresis (ABI Prism 3,100). The retrieving sequences were edited and aligned via the EditSeq and MegAlign programs (DNASTAR, Inc.; Madison, Wisconsin). Homology searches were conducted against the nonredundant (nr) NCBI GenBank database. Alignment was performed by Cluster W method and the phylogenetic tree was constructed in the MegAlign program.

RESULTS AND DISCUSSION

$TCID_{50}$ assay revealed different responses of each Ad serotype to cell lines. Ad41 is the most difficult one to culture on these cell lines. Only 293A was sensitive enough to permit the propagation of this serotype demonstrating

CPE (Figure 1). A low level of CPE for Ad41 was observed on HepG2 cells towards the very end of incubation. In contrast, Ad15 has a broad host-range and showed infectivity on all four cell lines within the first 24 hours of inoculation. Cell line 293A was generally 2 to 5 logs more sensitive to Ad serotypes tested than HepG2 and HuH7 (Figure 1). A549 supported the replication of Ad2 and Ad15 at a higher rate than HepG2 and HuH7, but it was insensitive to Ad41 tested. HepG2 and HuH7 were permissive for propagation for most serotypes tested at a lower rate. Only Ad41 did not display CPE on HuH7. The standard error for each data point collected in the experiment is approximately 10% or less. The serotype specific affinity to different cell lines had previously been shown by Zhang *et al.* (2003). This discrepant may be attributed to the quantitative difference in cellular receptor and viral hexon or fibre expression. Additionally, the Ad E1A protein is a trans-acting transcriptional regulatory factor necessary for transcriptional activation of early genes. E1A together with E1B are capable of transforming primary

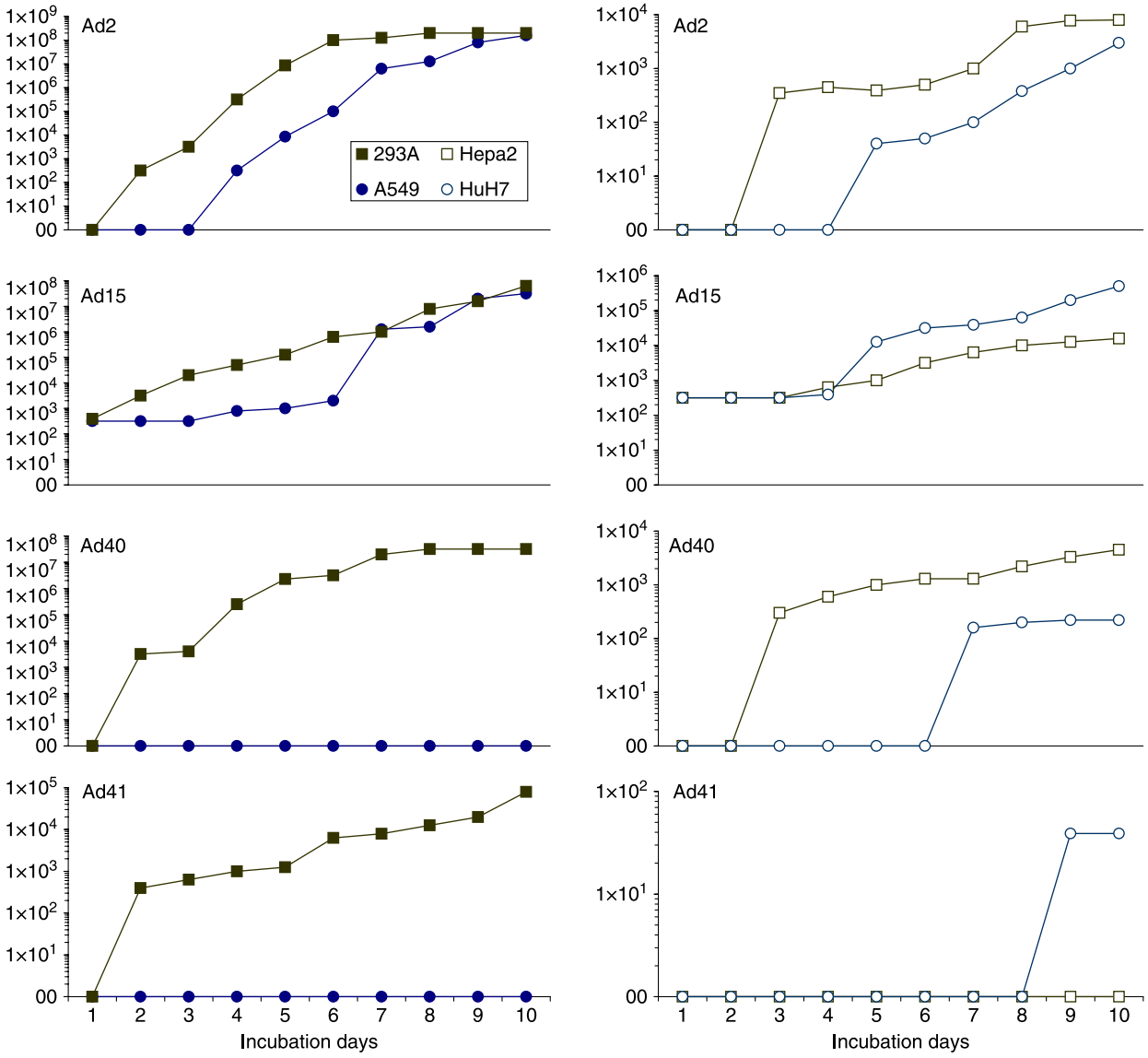

Figure 1 | $TCID_{50}$ assay of cell lines sensitivity to different serogroups of adenovirus. $TCID_{50}\,ml^{-1}$ was plotted on the log scale and infectivity was followed for ten days.

Table 1 | Plaque assay of adenoviruses in sewage using 293A and A594 cell lines

Sample source	Sampling date	Sample type	No. sample tested	Positive detection on 293A	Positive detection on A549	Plaque count (pfu/100 ml)	Detection limit (pfu/100 ml)
Orange county sanitation	October–December 2003	Mixed primary and secondary effluent	10	2 of 10	0 of 10	10–30	10
Irvine ranch water district	May–October 2005	Primary effluent	11	5 of 11	0 of 11	11–233	3
Orange county sanitation	6 July 2005	Primary effluent	1	1	0	11	3
LA sanitation	4 October 2005	Primary effluent	1	1	0	44	3

cells to sequester cellular machinery and alter the intracellular environment to favour viral replication. 293A is E1A/B transformed cell line, which may explain why the cell line is more susceptible and supportive to adenoviral infection. Based on these infectivity results, 293A and A549 were chosen for further assay of human Ads in the environment.

Cell line 293A and A549 were first used for plaque assay of Ad in sewage. Table 1 shows that viral plaques were detected on 293A cell line. More than half of the primary sewage effluents from southern California wastewater facilities produced viral plaques. The plaque counts were up to 233 pfu/100 ml. The detection rate was lower for samples containing mixed primary and secondary effluents, only 2 of 10 samples were positive. It is important to note that the detection rate presented here was limited by the volume of sample used. Only 10 ml of sewage sample was used for testing in 2003, 30 ml of sewage was used for later samples. The detection limit for mixed primary/secondary sewage was 10 pfu compared with 3 pfu for primary sewage (Table 1). The higher detection limit and the secondary treatment may have contributed to the lower plaquing rate in the mixed primary/secondary sewage effluents.

The plaque assay described above was then applied to 466 environmental water samples collected from various environments. The sample volumes ranged from 500 ml to 10 litres with concentration factors between 200 and 1,000 times. A fraction of the samples were positive for human Ads by PCR assay (Choi & Jiang 2005; Surbeck *et al.* 2006; Jiang *et al.* 2007*a*,*b*). However, infectious Ads were rarely detected by the plaque assay using either cell lines. Of all the samples tested, only samples from five sites were confirmed to have viral infection on 293A cells. Since the sensitivity of plaque assay is highly dependent on serotypes in addition to cell lines, the infected cells were extracted and used for cloning and sequencing to identify the Ad serotypes.

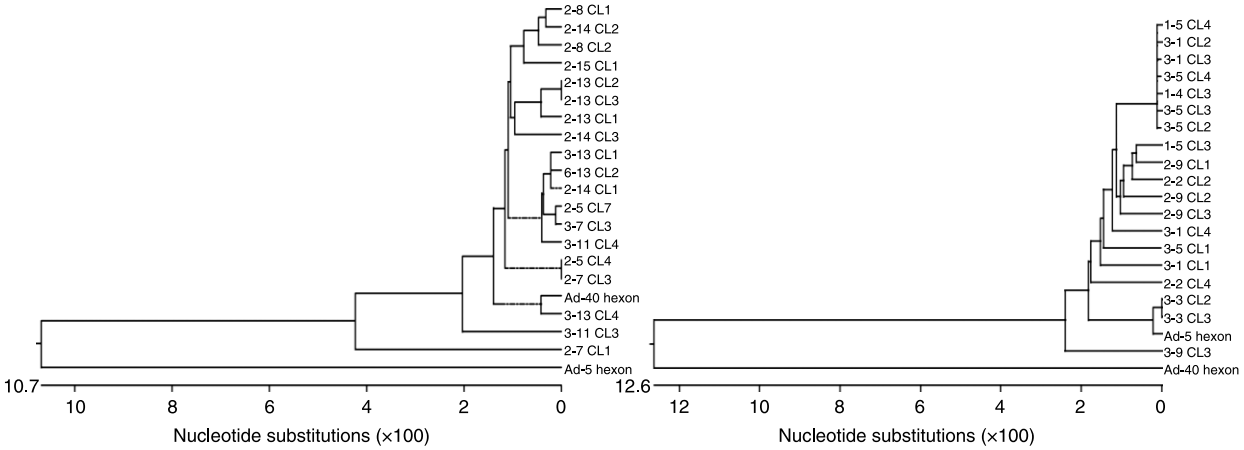

Figure 2 | Phylogenic relationships of environmental adenoviral hexon genes with adenovirus serotype 40 and serotype 5. The reference hexon gene sequences were retrieved from GenBank. Each environmental clone was designated with its sampling location, number of attempts at isolation and clone numbers. All clones were retrieved from four sites in southern California labelled 1, 2, 3 and 6.

Sequencing analysis of PCR amplicons retrieved from cell cultures showed that the hexon genes were aligned with Ad40 and Ad5 sequences from the GenBank (Figure 2). This result is in agreement with the clinical reports that Ad40 is the primary serotype shed in human faeces. Ad5 causes persistent infection in human populations. Shedding of this virus occurs long after initial infection (Jiang 2006).

Overall, the result of this study was that 293A cell line seems best suited for environmental assessment of human Ad contamination. Interestingly, Brown & Petric (1986) demonstrated that 293 cells are useful for the isolation of human Ad and certain enteroviruses from both respiratory and stool specimens. A549 cell used in this study was in higher passage numbers than 293A cell, which may also explain why the sensitivity of this cell line to environmental Ad isolation is lower. Furthermore, plaque assay in the environment may not be the sensitive method for detection of the infectious viruses. Adenovirus in the environmental samples may be stressed or injured, and thus less sensitive to plaque assay conditions. In addition, since plaque assay is a morphological assay, the viral pathogen of the plaque assay should be confirmed with other molecular approaches or immunoassays. Plaque assay combined with molecular detection methods should provide better sensitivity and specificity for environmental samples. We will also explore cell culture combined with immunoassay for detection of early-transcribed viral protein to improve specificity of detection for environmental samples. The outcome of this research is the first step to understand the suitable cell lines and dominant Ad serotypes among the environmental samples. Therefore, it is an important contribution to our effort in environmental monitoring of viruses in water.

CONCLUSIONS

The results of this study showed that different cell lines have various sensitivities to different Ads. Among the four cell lines evaluated, 293A and A549 were the most sensitive to enteric Ad40 and Ad41. The detection rate of infectious Ads in the southern California aquatic environment was fairly low using plaque assay. Viable Ads can be isolated from 10–30 ml of sewage samples through the plaque assay. Adenovirus serotype 40 and 5 were the two serotypes recovered from environmental samples. Combining 293A cell line with a molecular diagnosis method will be a sound future direction for viral monitoring in waters.

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